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14. ABSTRACT

In order to characterize the TIC population, we are developing a unique lentiviral barcode strategy to examine the growth kinetics and lineage of tumor cells. These studies are based on the hypothesis that TICs represent a limited subpopulation in breast cancers that give rise to tumor heterogeneity, and that these cells are intrinsically resistant to radiation and chemotherapy. In the first 12 months of the award period, we have successfully completed the first of our two specific aims.

15. SUBJECT TERMS
cancer stem cell, lineage tracing, lentiviral barcoding

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Introduction

The cancer stem cell theory posits the existence of a subset of tumorigenic cells with self-renewal potential and the ability to wholly reconstitute original tumors by lineage restriction. Although the expression of surface markers such as CD29/CD24 for mice and CD44/CD24 in humans have been employed to identify highly enriched, albeit heterogeneous populations of TICs, no lineage-tracing experiments have been performed to date to trace the fate of TICs. To characterize TIC growth kinetics in mouse mammary tumor cells transplanted into syngeneic recipient mice containing an intact immune system, we proposed developing a lentiviral barcoding system as a means of genetic lineage tracing of cell subpopulations sorted by surface marker expression. I will list and discuss the milestones reached in the first 12 months of the fellowship in this Annual Report.

Body

Task 1. Optimization of the multiplicity of infection (moi) of pGIPZ lentivirus in tumor cells. In order to faithfully model the propagation kinetics of the integrated barcodes, we need to determine the optimal multiplicity of infection (moi) to ensure that no more than one copy of lentivirus is integrated per cell, and that lentiviral infection efficiencies are not dependent on surface marker expression utilized in our antibody-based sort. These studies will be initially done in the low-attachment mammospheres culture conditions (month 1-3).

1a. Construct new barcode virus by subcloning barcode sequences into pGIPZ vector (month 1-2). We decided to pursue another lentivirus vector (FU-CRW, courtesy of Dr. Li Xin), which carries the mRFP transgene instead of the GFP transgene in the original pGIPZ vectors. The rationale for switching vectors is two-fold- 1) mRFP expression is less toxic to mammalian cells than GFP, and 2) using RFP frees up the FITC fluorescence channel for the flow cytometer. So far I have successfully engineered 4 recombinant FU-CRW lentivirus with unique genetic barcodes. Each lentivirus has been sequence-verified. ***1b. Optimize infection conditions (e.g. cell density per well, optimal moi) (month 2-6).*** Through optimization trials, we determined that optimal condition for lentiviral infection of the p53 null mammary tumor cells is overnight incubation roughly at moi = 10 with 500,000 cells. The cells are temporarily cultured in F-12 media supplemented with bFGF, EGF and B27 Serum-Free Supplements (Invitrogen). ***1c. Optimize quantitative PCR assays to detect barcode abundance in infected cells (month 2-6).*** Genetic barcodes were inserted in the FU-CRW vectors and verified by TaqMan chemistry probe and primer sets (IDT DNA). Probes are highly sequence-specific (see Figures 1a-d; Supporting Data) ***1d. Optimize infection conditions of no more than single virus integration per cell (month 2-6)*** Because of initial concerns that immuno-sorted cell fractions may differ in their susceptibility to virus integration, I decided to pursue a slightly modified protocol in labeling the tumors with genetic barcodes. In the modified protocol, single cells from the parental tumor are infected

with unique barcode viruses and propagated as separate "sublines". These tumor sublines should be identical to each other except for the integrated unique genetic barcode. In this modified protocol, individual tumor sublines can be propagated and immuno-sorted prior to each round of transplantation (Figure 3a-e). To ascertain that cells from the tumor sublines harbor no more than one single barcode integration per cell, we performed quantitative qPCR with barcode-specific probes and normalized the cycle threshold values (C_T) of the barcode qPCR with the C_T of an endogenous gene locus (beta-casein). Our initial results indicate that all tumor sublines tested have no more than 1 barcode integrated per cell. ***1e. Determine infection efficiencies of antibody-sorted cell fractions (month 6-12)- 20 mice will be used for tumor transplantation.*** The modified protocol has described above has obviated the need to determine the efficiency of lentivirus infection between immuno-sorted fractions.

Task 2. Optimization of tumor transplant conditions. The p53 null stochastic tumor model has been well characterized by our laboratory. At present, we have developed a p53 null tumor bank, which can be thawed and transplanted into recipient mice as needed.

2a. Optimize minimum cells needed for transplantation to reconstitute tumors (month 13-24) - 80 mice will be used in transplantation procedures. Preliminary optimization trials indicate that as little as 5,000 cells per barcoded tumor subline can reconstitute tumors within 4-6 weeks after transplantation (Table 1; Supporting Data). ***2b. Optimize antibody concentration and fluorophore combinations for FACS analysis (month 13-18) - 80 mice will be used in transplantation procedures.*** I am using the Becton-Dickinson Aria II flow cytometer for the FACS analysis. The Aria II has four lasers (UV-355nm, blue-488nm, yellow/green-561nm, red-638nm). I have successfully utilized the combination of AlexaFluor 350 and FITC for immuno-sorting of the tumor sublines (Figure 2a-e; Supporting Data). Because the mRFP emission occupies the same spectrum as the PE flurorescence channel, I am currently exploring ways of using magnetic beads separation method to remove Lin+ cells in the tumor mixture. ***2c. Optimize mixing ratios of sorted cell fractions for transplantation (month 13-24) -120 mice will be used in transplantation procedures.*** The experiments have not progressed to this point and will commence in Year 2.

Task 3. Development of the statistical model to analyze the distribution of barcode abundance in tumor outgrowths. 3a. Consultation with biostatistician, Dr. Susan Hilsenbeck at the Baylor College of Medicine (listed as a collaborator) (months 1-18) The experiments have not progressed to this point and will commence in Year 2.

Key Accomplishments

- Constructed new barcode virus by subcloning barcode sequences into pGIPZ vector (month 1-2)
- Optimized infection conditions (e.g. cell density per well, optimal moi) (month 2-6)
- Created/optimized quantitative PCR assays to detect barcode abundance in infected cells (month 2-6)
- Optimized infection conditions of no more than single virus integration per cell (month 2-6)
- Determined infection efficiencies of antibody-sorted cell fractions (month 6-12)- 20 mice will be used for tumor transplantation
- Optimized minimum cells needed for transplantation to reconstitute tumors (month 13-24) - 80 mice will be used in transplantation procedures
- Optimized antibody concentration and fluorophore combinations for FACS analysis (month 13-18) - 80 mice will be used in transplantation procedures.

Reportable Outcomes

-None to include

Conclusion

In conclusion, milestones proposed in the original award have been met in timely fashion in the first 12 months. I have developed the qPCR assays, optimized the antibodies and flurophore combination, and established 4 barcoded tumor sublines for lineage-tracing experiments.

Appendices

-None to include

Supporting Data

Figure 1. Quantitative PCR with specific probes and primers detecting unique genetic barcodes. For comparison, genomic DNA from roughly 5,000 cells from each tumor sublines were isolated and quantitated by real-time PCR using sequence-specific TaqMan probes. A) BLV-Kpn1; B) BLV-Hind3; C) BLV-42; D) BLV-61; E) BLV-151.

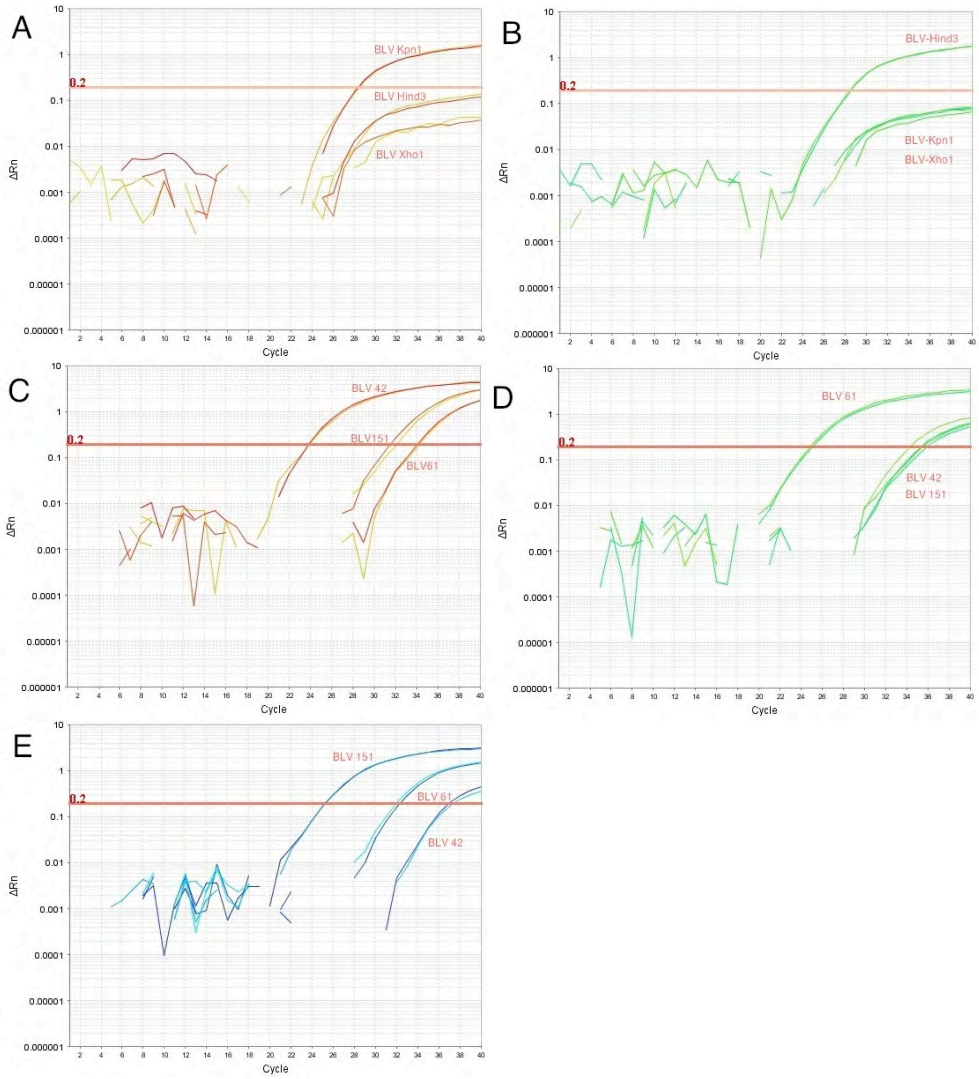

Figure 2a. FACS profile for T7 tumor subline BLV-Hind3 stained with CD29-FITC and CD24-biotin (+SAV-AF350)

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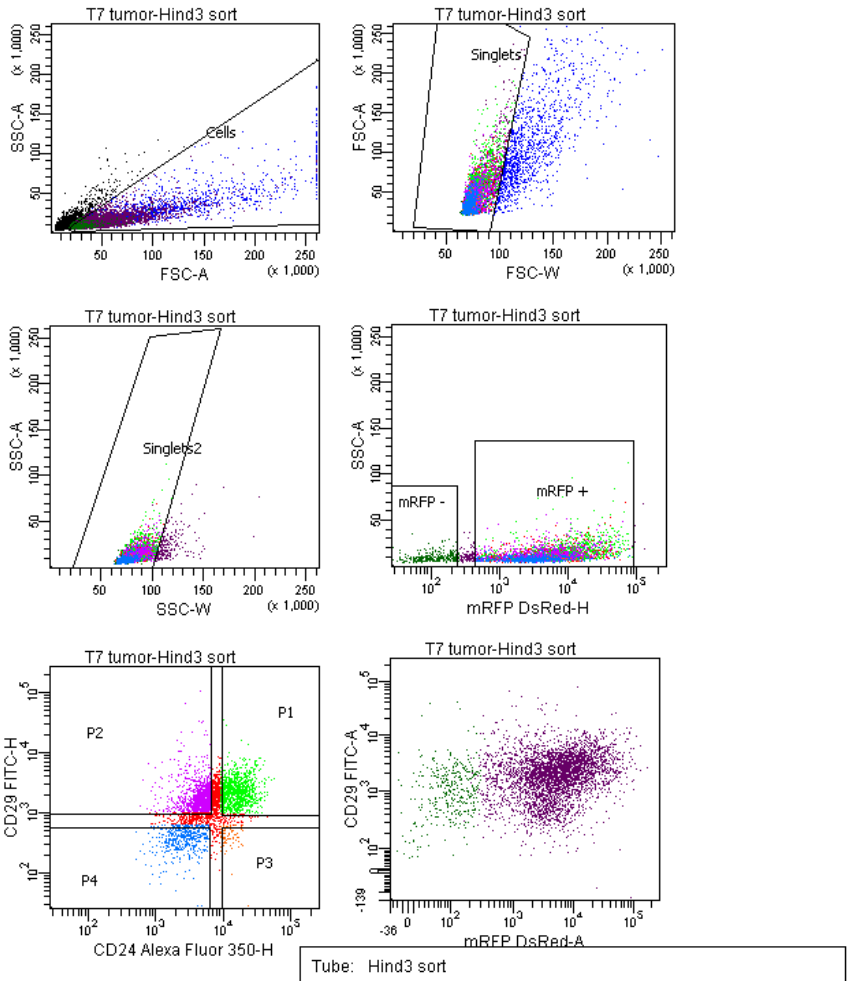

Tube: Hind3 sort

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Cells	5,619	56.2	56.2
Singlets	4,364	77.7	43.6
Singlets2	4,111	94.2	41.1
mRFP +	3,637	88.5	36.4
P1	1,085	29.8	10.8
P2	1,017	28.0	10.2
P3	76	2.1	0.8
P4	484	13.3	4.8
mRFP -	355	8.6	3.6

Figure 2b. FACS profile for T7 tumor subline BLV-Kpn1 stained with CD29-FITC and CD24-biotin (+SAV-AF350)

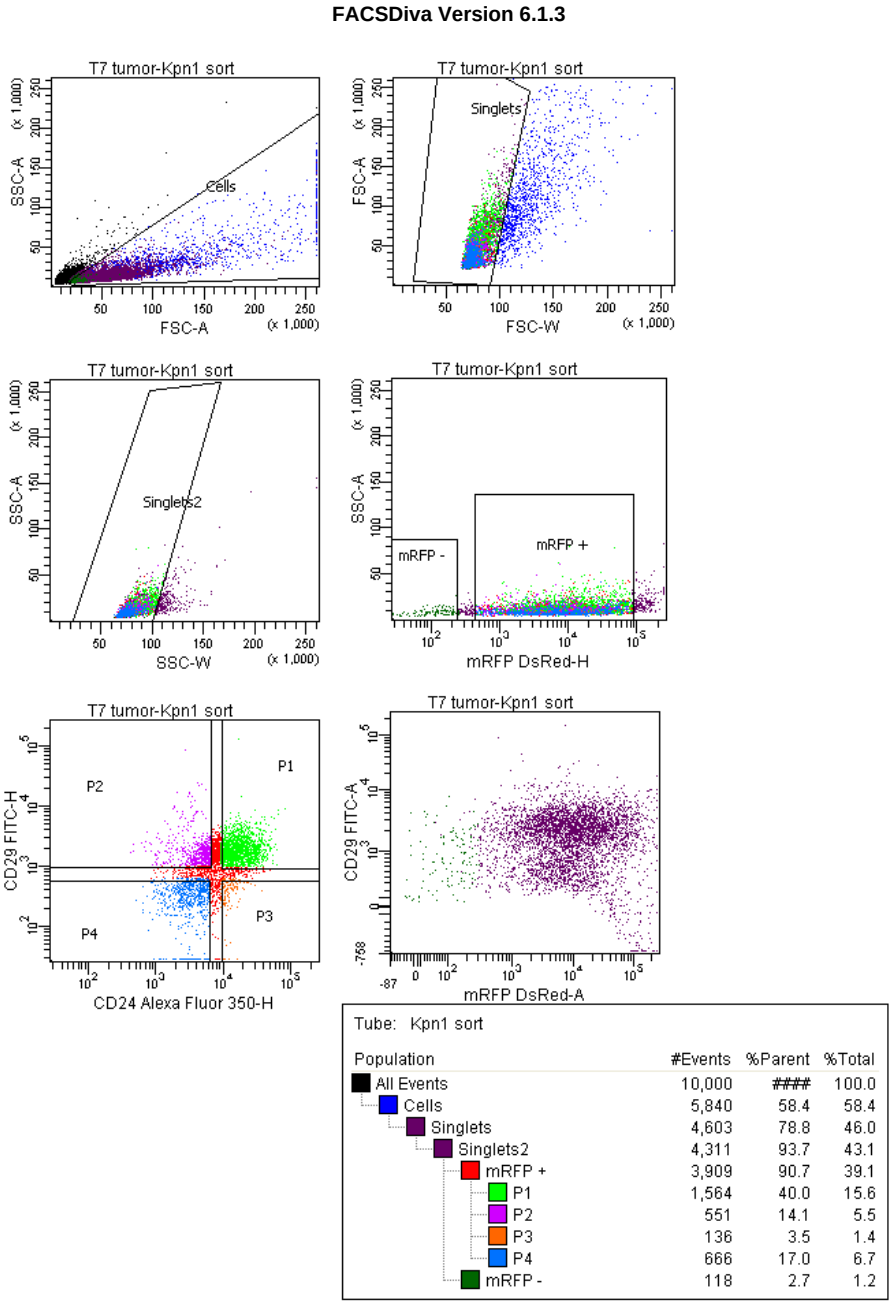

Figure 2c. FACS profile for T7 tumor subline BLV-42 stained with CD29-FITC and CD24-biotin (+SAV-AF350)

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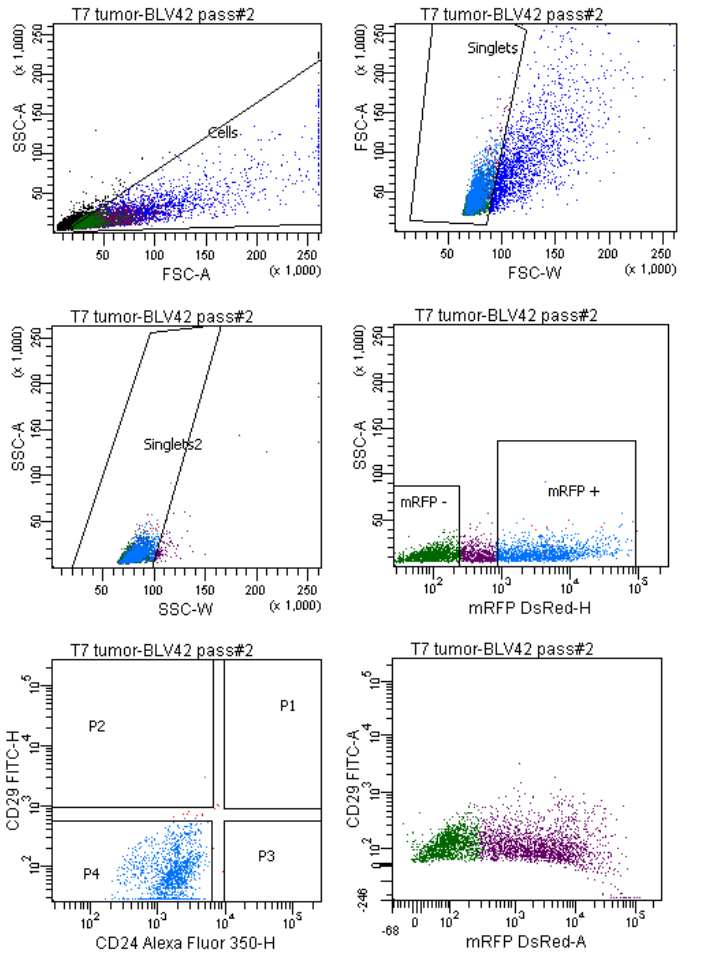
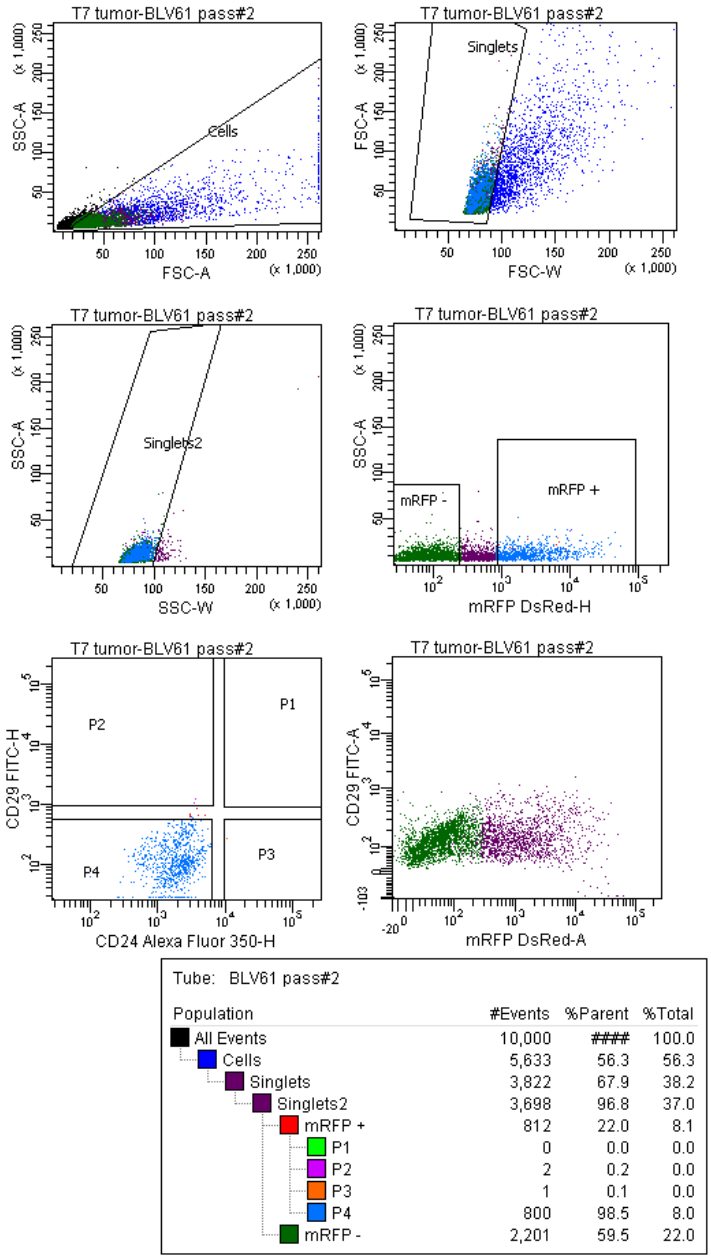


Tube: BLV42 pass#2

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Cells	5,302	53.0	53.0
Singlets	3,491	65.8	34.9
Singlets2	3,384	96.9	33.8
mRFP +	1,435	42.4	14.4
P1	0	0.0	0.0
P2	1	0.1	0.0
P3	0	0.0	0.0
P4	1,422	99.1	14.2
mRFP -	1,418	41.9	14.2

Figure 2d. FACS profile for T7 tumor subline BLV-61 stained with CD29-FITC and CD24-biotin (+SAV AF350)

FACSDiva Version 6.1.3

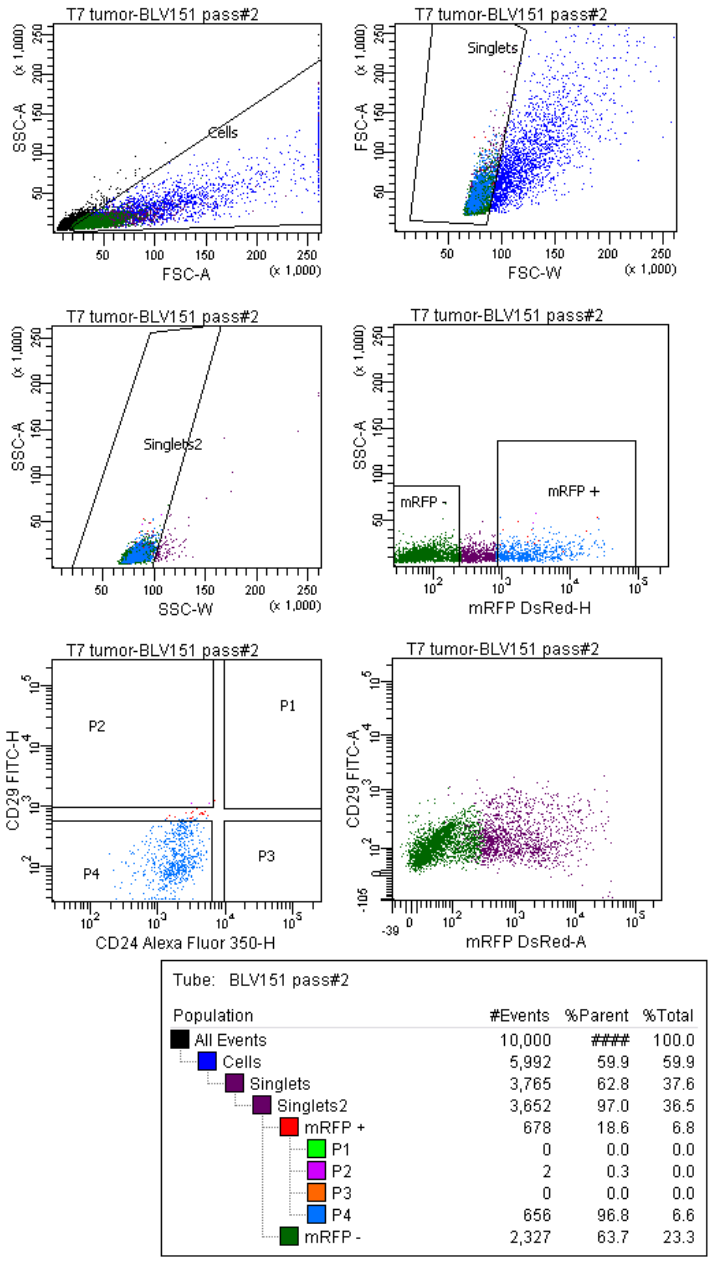


Tube: BLV61 pass#2

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Cells	5,633	56.3	56.3
Singlets	3,822	67.9	38.2
Singlets2	3,698	96.8	37.0
mRFP +	812	22.0	8.1
P1	0	0.0	0.0
P2	2	0.2	0.0
P3	1	0.1	0.0
P4	800	98.5	8.0
mRFP -	2,201	59.5	22.0

Figure 2e. FACS profile for T7 tumor subline BLV-151 stained with CD29-FITC and CD24-biotin (+SAV-AF350)

FACSDiva Version 6.1.3



Tube: BLV151 pass#2

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Cells	5,992	59.9	59.9
Singlets	3,765	62.8	37.6
Singlets2	3,652	97.0	36.5
mRFP +	678	18.6	6.8
P1	0	0.0	0.0
P2	2	0.3	0.0
P3	0	0.0	0.0
P4	656	96.8	6.6
mRFP -	2,327	63.7	23.3

Table 1.

Date	Cells injected/ mouse	Tumor outgrowth/ Mice injected
1/8 - 2/10/2010	50,000 cells	6 tumors/ 7 recipient mice
2/10 to 3/22/2010	25,000 cells	6 tumors / 6 recipient mice
3/23 to 4/30/2010	5,000 cells	3 tumors / 4 recipient mice